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VOLUME IV NUMBER 2

STAIN TECHNOLOGY

APRIL, 1929

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SUGGESTIONS TO AUTHORS SUBMITTING MANUSCRIPT

Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

To be accepted for publication, manuscript should be original typewritten copy (not carbon copy) either double or triple spaced, with wide margins. Special attention should be given to the headings of tables and to legends for illustrations. These in all cases should be complete in themselves, so as to make the table intelligible to anyone studying it, without consulting the text of the article. In the case of complicated tables, typewritten manuscript is not absolutely required, provided all words and figures are carefully written or printed in a legible hand. All references to literature must be carefully verified and in ordinary cases the complete title and reference should be given.

Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

All illustrations should accompany the manuscript and should be numbered with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. Line drawings must be made in India ink. Photographs for half-tones must be on paper giving black and white tones and should preferably have a glossy finish.

Authors are especially urged to make all staining formulae definite and accurate. If known to the author, the dye content of the stain should be given and the exact dye used should be specified. If this information is not known, the name of the manufacturer should be stated and that of the stain itself exactly as worded on the label. Certified stains should be specified by certification number.

STAIN TECHNOLOGY

VOLUME IV APRIL, 1929 NUMBER 2

PROGRESS IN THE STANDARDIZATION OF STAINS

DEFINITENESS IN STAINING FORMULAE

Three years ago[1] the standardization of staining solutions was discussed under this heading, and it was urged that formulae be given in some such form as the following:

Basic fuchsin (88%)	0.6 g.
	(representing 0.53 g. actual dye)
Ethyl alcohol (95%)	10. cc.
Distilled water	100. cc.

Such a formula as this was recommended because of its being more definite than the sort of formula frequently occurring in the literature, such as "one part alcoholic solution of fuchsin, 10 parts of water." There is no question but that the former type of staining formula is more definite than the latter and everyone publishing a staining solution is still urged to use this more definite form.

On the other hand, even the staining formula recommended in this earlier article is not as definite as it might be. What, for instance is intended by basic fuchsin? There are, as explained on a later page (55) of the editorial referred to above, three primary types of basic fuchsin, and stains purchased from the different supply houses may be various mixtures of these three compounds. A staining formula cannot be regarded as absolutely precise unless it tells which of these types is most nearly represented by the sample actually employed and what the actual dye content of that sample was. This information, to be sure, is seldom directly available to the user of any stain and in many cases is not known even to the manufacturer. It is, therefore, undoubtedly expecting too much to hope that the users of stains will publish the exact chemical nature of any dye they employ. At the time of this earlier editorial it seemed absolutely out of the question to expect any such procedure to be followed.

At the present time, however, some information of the sort is entirely possible, because over forty of the most common biological stains have now been put on the certification basis. In the case of

[1]Standardization of Staining Solutions. Stain Tech. **1**, 51–3. 1926.

these certified stains the manufacturer is expected to place on the label the Colour Index number of the dye and the certification number of the sample, as well as the total dye content when known. If anyone using a certified stain gives these two numbers and mentions the dye content in publishing his formula it will be possible for anyone else to identify the dye that he has actually employed. The Colour Index number does not in every instance tell the exact chemical formula of the dye, but it does at least tell the type of the dye so far as it is ordinarily known to the manufacturer. The certification number, moreover, identifies the particular batch upon the records of the Stain Commission so that one can obtain even more definite information as to the exact nature of the dye by writing to us about it. In other words if the formula is given in the form recommended above, and the dye is definitely specified by its Colour Index number and certification number, a fair degree of definiteness is certainly accomplished.

In the case of many stains, to be sure, this cannot be done. Not all biological stains have been put on the certification basis, and the certification system has so far been applied to stains of American manufacture only. The dyes that are not certified, of course, bear no certification number and it is rare that they are denoted on the label by Colour Index number (or even its German equivalent, the Schultz number). The best one can do in the case of some stain inadequately labelled is to give such information as is furnished by the label. Anyone publishing the results of staining technic, who has employed other than a certified stain in his work, should, therefore, give all the information obtainable from the label—in other words the name of the dye, exactly as printed on the label, together with any shade designations or other qualifying information furnished by the manufacturer. He should also give the name of the manufacturer as shown on the label. This latter information has some times been withheld for fear it will be furnishing the manufacturer with free advertising. The matter should not be looked at in this way, however; the manufacturer's name should be regarded as necessary for the identification of the product. Even this information is not complete, but it is often the best available.

A brief glance at textbooks on microscopic technic will show that this information is seldom furnished. Such formulae as the following are very common:

Acid fuchsin	10 g.
Absolute alcohol	33 cc.
Water	66 cc.

or the following:

Methylene blue, sol. sat. in water	40
Eosin, 0.5 per cent in 70% alcohol	20
Distilled water, or glycerin	40

Such formulae are certainly extremely indefinite. Of the three dyes in these two formulae, selected at random by glancing thru the pages of a couple of books on technic, the first names have a rather vague meaning to the dye chemist. Different lots of methylene blue vary considerably with the extent of oxidation in the process of manufacture, while acid fuchsins vary tremendously. Eosin is a more definitely known dye, assuming the formula intends to refer to eosin Y, as is probably the case; but even in regard to this stain it must be noticed that a 5% solution may vary in actual strength according to the dye content of the sample employed. Neither of these formulae say anything about the dye content to be preferred in the case of any of the dyes; while in the latter a saturated solution of methylene blue might vary considerably in strength according to the amount of impurities present in the lot actually used. In the case of this latter formula, where a basic and an acid dye are combined, it is exceptionally necessary to specify exact dye contents and the strength of the solutions employed, because acid and basic dyes combine in definite proportions and it is important that suitable relative proportions of each be furnished.

There is, to be sure, some excuse for the writer of a textbook who publishes an indefinite formula. His formulae is usually quoted from the literature and he has no way of knowing what actual dye was employed by the originator of the formula. Is it, however, too much to ask that a textbook writer state whether or not he has employed some particular formula himself, and if he has, mention the identity, and if possible the dye-content, of some dye sample with which he has obtained good success?

In the case of authors publishing original investigation, there is less excuse, for any such investigator should know how his dyes are labelled. As this seems to be a very important point, it has been decided not to accept papers for this journal unless such information as this is furnished in regard to all the dyes named. Furthermore, for the sake of its educational value, it is planned in the abstracts of technic published in the last section of each issue to state whether or not an author has given definite information as to the source or nature of the dye employed. It is hoped that in these two ways the Commission can bring about an improvement in the definiteness of staining formulae appearing in the literature.

H. J. Conn

Stains Recently Certified

Following the policy begun in the last issue, a list is here given of the batches of stain recently certified. The following list covers three months, beginning with the first stain approved later than the last one listed in January.

Stains Certified December, 1928 to February, 1929*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Brilliant green	NBg 3	94%	For typhoid enrichment media	Jan. 15, 1929
Jenner's stain	NJr 2	——	As blood stain	Jan. 15, 1929
Methylene blue tablets	NA 2	——	For reductase test in milk	Jan. 15, 1929
Methylene blue (crystals)	NA 3	86%	As bacterial and histological stain	Feb. 1, 1929
Methylene blue (for bacilli)	CA 7	86%	As bacterial stain	Feb. 4, 1929

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples here listed may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its practical value.

THE HISTORY OF STAINING[1]

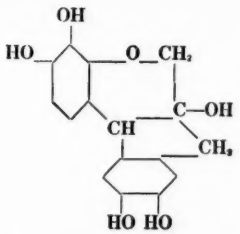
LOGWOOD DYES

H. J. Conn, *Geneva, N. Y.*

Prepared with the assistance of S. I. Kornhauser and F. W. Mallory.

Except for the cochineal derivatives, logwood extract was the first of the important modern stains to be employed in histology. Certain other natural dyes, such as madder and indigo, had been used earlier, but they are of little significance in discussing the history of staining, because none of them nor even alizarin, the derivative of madder, are of any appreciable significance in these days of synthetic dyes. Hematoxylin, on the other hand, still continues a very important stain, and it has played an interesting part in the history of staining.

Hematoxylin is a purified form of the dye present in logwood extract. It is a glucoside, the chemical formula of which is considered to be

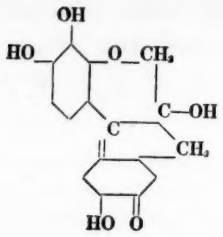


It is manufactured by extracting commercial logwood extract (aqueous) with ether and subsequent crystallization with water. The original hematoxylins employed by biologists were very crude, some of them scarcely more than an aqueous extract of logwood. Gradually the method of refinement has been improved, yielding a product quite free from the extraneous matter present in logwood. It is only within comparatively recent years, however, that the process has been so standardized as to yield a comparatively uniform product.

Hematoxylin itself is not a dye, and in order to use it as a stain it must be oxidized into the product hematein[2] which has the following composition:

[1]The preceding papers of this series have appeared as follows: A preliminary note. H. J. Conn, Stain Tech. **2**, 126–7. 1927. The pioneers in staining. H. J. Conn, Id. **3**, 1–11. 1928. Cochineal dyes. H. J. Conn and S. I. Kornhauser, Id. **3**, 110–121. 1928.

[2]This compound must not be confused with hematin, the colored constituent of red blood corpuscles. The chemistry of hematin is not entirely known, but it is a nitrogenous compound that bears no relation to the hematein derived from logwood



Hence, in any staining procedure involving hematoxylin, the solution must be oxidized before use, probably with some oxidizing agent present in solution; or else it must be employed for staining tissue mordanted with some metallic salt capable of bringing about the necessary oxidization during the process of staining. This is the reason for the well-known alum-hematoxylin formulae, such as those of Böhmer, and Delafield. Mayer insists that alum-hematoxylin formulae should be classed as solutions of hematein rather than of hematoxylin.

Hematein can be purchased from dealers in biological stains. Its commercial production does not, however, at the present time seem to have been given the amount of attention that has been given to the manufacture of hematoxylin itself. It may, therefore, happen that a commercial sample of hematein is much more crude than one of hematoxylin, altho the former compound is supposedly derived from the latter.

Very similar to hematoxylin, altho of less histological importance is the dye, brazilin, derived from Brazil wood. It is a lower homolog of hematoxylin, containing one less hydroxyl group, and its oxidation product, brazilein is similarly homologous to hematein. This dye has played little part in the history of staining; but recent work suggests that it may have important application in cytology.

Earliest Use of Hematoxylin

As in the case of carmin, there is some uncertainty as to the first date when hematoxylin was employed as a biological stain. Gierke (1884) is undoubtedly correct in stating that the first biologist thus to use it was Waldeyer (1863). Waldeyer, however, was not very successful in this, as he employed a plain aqueous extract of logwood, and had not learned the technic of satisfactory mordanting before applying the dye. He tried to employ the dye chiefly for staining the axis cylinders of nervous tissue, but the results were so questionable that he gave it up in favor of the extract of Alkanna roots.

Alum Hematoxylin

Two years later Böhmer (1865) proposed a successful method of employing the dye. His formula has much historical interest, therefore, altho it is no longer employed exactly as proposed by Böhmer. A translation of his description of this staining formula (*l. c.*, p. 549) follows:

"Staining of tissue can be well accomplished with hematoxylin and alum. Pure hematoxylin is dissolved in absolute alcohol (. . . .) and 2 or 3 drops placed in watch glass that is filled with a solution of alum in distilled water (. . . .) Sections are left in this for a day; then passed thru absolute alcohol, tartaric acid in alcohol, absolute alcohol, turpentine, ricinus oil. Alcoholic extract of logwood instead of hematoxylin does not work. Preparations treated in chromic acid or potassium dichromate or with copper sulfate or certain other metal salts are colored blue in plain aqueous hematoxylin."

Böhmer's formula was stated in terms of apothecary's weight, but is generally converted into the metric system and given essentially as follows:

Solution 1		Solution 2	
Hematoxylin (crystallized).....	0.35 g.	Alum.....	0.1 g.
Absolute alcohol.....	10 g.	Distilled water.....	30 g.

A few drops of the first solution are added to the second, the amount employed varying the concentration desired.

The success of Böhmer where Waldeyer had failed was due to the fact that he realized that alum was widely employed as a mordant in the dyeing industry, and knew that logwood had a stronger dyeing power in its presence than when it was absent. He made use of this practical knowledge to much advantage; and, altho his formula is not used without modification today, the principle which he introduced in the use of alum is widely followed.

There seems to be little question but that Böhmer was the first to employ hematoxylin successfully. Nevertheless Arnold (1872) states that this dye was probably first used by C. F. Müller in his investigations of the cornea. This work of Müller's has not yet been located and it is a question whether it actually precedes that of Böhmer.

Böhmer also found that preparations treated in chromic acid, potassium bichromate, copper sulfate or certain other metal salts were stained blue by alcoholic hematoxylin diluted in plain water (instead of alum solution). Gierke mistakenly credits Frey (1868) with this discovery. Actually Frey quotes the procedure from Böhmer, using Böhmer's own words and citing the very page in the

latter's paper from which the quotation is taken. It is plain, therefore, that Frey had no intention of claiming credit in the matter, and it seems strange that Gierke should not have noticed that Frey was quoting someone else in the passage in question.

A little later than this Merkel (1872) employed hematoxylin for staining the muscles of animals in polarization studies, but does not definitely specify his technic. In the same year Arnold (1872) wrote especially enthusiastically concerning the value of hematoxylin as a stain. He gave rather more detailed instructions for preparing a satisfactory alum-hematoxylin solution than previously in the literature, but does not contribute anything materially new in his formula. His formula consists of alum and logwood extract ground up together and dissolved in water and subsequently mixed with 75% alcohol.

At about this time Kleinenberg proposed a formula for hematoxylin, containing alum and calcium chloride dissolved in 70% alcohol, which figures considerably in the literature of the period. The exact date of the first publication has not yet been found, the usual citations being 1876, 1878, or 1879, altho Tait (1875) refers to the formula as tho it were well known at the date of his paper (see below). An easily available reference to an early publication of this formula is Kleinenberg (1879). The chief objection to Kleinenberg's formula seems to have been indefiniteness, neither the quantity of alum nor that of the dye being specified, so that its exact duplication by others was difficult.

Tait's paper (1875) contains some interesting comments on the general situation as to staining in his day, together with some conclusions on the theory of staining that are about the opposite of those held today. Certain quotations from his paper are quite interesting:

"Of substances which act as tissue stains, I find that there are two classes, one which simply colors the whole mass exclusive of fat and the other which discriminates either regularly or irregularly. Fat is stained by alkannin only. In the first class may be placed all the anilin colors and most of the solutions of carmine, also several other coloring matters less known. This class is comparatively useless for investigation and all, save carmine, can be used only when it is desirable to keep structures from disappearing entirely from view, as when they are mounted in some terebinthinate, such as balsam, dammar, colophonium, sandarak, etc. But as all these vehicles are quite incompatible with the more delicate demonstrations, I have quite discarded them, and with them all the stains belonging to the first class, almost including carmine. This last substance is so uncertain, so liable to capricious alteration, and its action is so crude that I regard it as little better than anilin."

Tait then states that the second class of stains have the power of discriminating between nucleus and "corpuscle." Carmin shows this difference to a limited extent. He concludes: ". . . that the nucleus of the corpuscle is faintly more alkaline than the body of the cell, the general view, originating, I think with Dr. Beale, being that the nucleus is more acid than the body of the corpuscle." Tait mentions in support of this that ammonia carmin stains the nucleus better when the ammonia is first neutralized by HCl with the acid added till litmus solution is slowly decolorized; then he continues: "Therefore the best stains are litmus and red-cabbage, but they are the most difficult to work with. Hematoxylin is very good, but it is not adapted to the finest work and in the solutions of Klein and Kleinenberg it is very rough. The former adds a quantity of alum, which makes the solution soon deteriorate, and it is very erratic in its results, whilst the solution of Kleinenberg, containing calcium chloride and alum, seems to me to destroy the sections.

"My own experiments with logwood have been made with the ordinary extract sold by the druggists and the crystalline hematoxylin prepared by Eidenbenz of Dresden. Of the former, I made a strong watery solution with the aid of heat, filtered it, and added to it when cold about 10 per cent of pure spirit, or a drop of oil of cloves, to make it keep. A few drops of this poured on to a fresh section will stain it a pale brown in a few minutes and the addition of a few drops of a .004 per cent solution of nitric acid in distilled water will display the nuclei a faint cherry brown color, whilst the rest of the tissue becomes a cherry red. This is due to the fact that the faintly acid solution overcomes the faint alkalinity of the tissue and alters the color of the stain except in the nuclei where the greater alkalinity returns the color and prevents its alteration by the acid. Some practice is required not to use too much acid.

"Again, if a cherry-red mixture is made by adding my standard solution of nitric acid (4 per cent) carefully to a quantity of the solution of the extract of logwood, the general tissue becomes cherry-red, whilst the nuclei restore the brown color to the stain which enters them. The same experiment can be repeated with Klein's solution. Let a section remain in that solution till it is shriveled, crumpled, hard, and almost black. Then put in a watch glass with some distilled water and add the standard acid solution drop by drop gently, moving the section about during the process till it begins to get limp and discolored. Then remove it and wash it in ordinary water, and it will be found that while the nuclei retain the brilliant pure color, the general tissue has become cherry-red or light brown."

It will be noticed that in one of the above quotations Tait refers to an alum-hematoxylin by Klein. Up to the present time no information is at hand as to what this formula was or when it was first proposed. It does not figure to any great extent in the literature, and probably contributed little, if any thing of importance, to the development of staining methods with this dye.

At about this period the important formula now known as Delafield's hematoxylin was proposed. Whether this formula was published by Delafield himself at the time is doubtful; the reference to its first publication, as generally given, is a note by Prudden (1885), who furnished the formula in response to an inquiry in an earlier number of the journal in which his note appeared. Evidently the formula was well known in certain laboratories at this date. It is prepared by adding to 400 cc. of saturated aqueous solution of alum, 4 g. of hematoxylin dissolved in 25 cc. of "strong" alcohol; this is allowed to stand 3 or 4 days exposed to air and light, then filtered; to it is then added 100 cc. glycerin and 100 cc. methyl alcohol. The solution is then allowed to stand until the color is "sufficiently dark," and subsequently kept in a tightly stoppered bottle, diluting considerably when used.

By this time, hematoxylin methods, other than those calling for alum in the staining solution, were coming into use; but it is well to follow up the later developments in regard to alum hematoxylin before considering the others. Renaut (1881) proposed a glycerin hematoxylin prepared as follows: An absolutely neutral glycerin "very syrupy" (e. g. 1260°) is saturated with potassium alum. Drop by drop a "concentrated" alcoholic solution of hematoxylin is added until the solution is "sufficiently" violet. The mixture is filtered and ripened in a flask covered with a piece of paper perforated with a few holes.

Subsequently Ehrlich (1886) (see also 1910, I, 599) gave a different formula for a gylcerinated alum-hematoxylin, which is of some importance:

Water	100 cc.
Absolute alcohol	100 cc.
Glycerin	100 cc.
Glacial acetic acid	10 cc.
Hematoxylin	2 g.

The hematoxylin is dissolved in the alcohol, the acid added to it, then the glycerin and water. Alum is added in excess. The solution is ripened in an open flask in the light until dark red.

The next most important development in an alum-hematoxylin was by Mayer (1891). Somewhat earlier Mayer (1880) had written at some length concerning hematoxylin in discussing the microscopic methods employed at the Naples Zoological Station. In this earlier paper, however, he laid most stress on the Kleinenberg formula, altho admitting its weakness. Mayer explained that by bringing alum and calcium chloride together in the Kleinenberg formula one gets a precipitate of gypsum; one would therefore do better to employ

aluminium chloride instead of alum. In the 1891 paper he gives one or two formulae embodying this principle, one of which he states to have been obtained from Kleinenberg's laboratory where its use had replaced the original formula. This formula is as follows:

$CaCl_2$	about 8 g.
$AlCl_3$	about 0.15 g.
Some KCl or NH_4Cl (according to the kind of alum desired)	
A little free HCl	
Alcohol (60–65%)	100 cc.

In this same paper (1891) Mayer points out that the ripening of hematoxylin is for the purpose of converting it into hematein, and that a ripened alum-hematoxylin solution is really a solution of hematein. In a still later paper (1899)[4] he even goes to the extent of listing all alum-hematoxylin formulae under hematein. He explains that hematein as well as hematoxylin can be obtained commercially and states that he prefers it as a basis of staining formulae. Two of his best known formulae, published in the 1891 paper, known as "hemalum" and "hemacalcium," both call for hematein in dry form. The latter of these two formulae is a substitute for Kleinenberg's hematoxylin, and is claimed by Mayer to be preferable. Its formula is:

Hematein	1 g.
$AlCl_3$	1 g.
$CaCl_2$	50 g.
Glacial acetic acid	10 cc.
Alcohol (70%)	600 cc.

The first two ingredients are ground together, the acetic acid and alcohol added; they are dissolved cold or warm. Last the calcium chloride is added.

Mayer's hemalum, given in this same paper, is another hematein formula, calling for aluminium in the form of alum instead of the chloride. For this formula 1 g. of hematein is dissolved in 50 cc. 90% alcohol by means of gentle heat, and is then added to a solution of 50 g. of alum in 1 litre of distilled water.

At this same time (1891) Mayer shows how ammoniacal hematein may be prepared in dry form; 1 g. hematoxylin is dissolved in 20 cc. distilled water and filtered; 1 cc. of ammonia (sp. gr. 0.873) is then added, and the fluid is placed in a dish large enough so that the level is only 0.5 cm. above the bottom, and is stood in a place free from dust to allow the water to evaporate.

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[4]This paper of Mayer's (1899) is extremely interesting in its comprehensive historical discussion of hematoxylin and carmine, giving the most important formulae proposed up to that time.

Hematoxylin With Chromium

The development of staining methods calling for the use of chromium compounds with hematoxylin is so well discussed by Mayer (1899, p. 199) that a translation seems desirable:

First employed by Böhmer (1865), who immersed the sections in aqueous hematoxylin after washing them with a chrom mixture; then by R. Heidenhain. The latter (1885) first used potassium bichromate, later (1886) monochromate, for the preparations held better with the latter. He placed the object for 12 to 24 hours in a ⅓% aqueous solution of hematoxylin, then for the same length of time in a 0.5% aqueous solution of potassium chromate, later thru alcohol and xylol into paraffin.—Apathy (1888), and later Plantner (1889) modified the first procedure of Heidenhain by dissolving the bichromate as well as the hematoxylin in alcohol and by carrying out the operations so far as possible in the dark. Series of celloidin sections are placed for 10 minutes by Apathy (1889) in his hematein solution (1% in 70–80% alcohol), are dried with blotting paper (so that the celloidin will not also be stained) and brought in the dark into 70% alcohol containing a few drops of 5% bichromate solution, for 5–10 minutes. . . .

Especially important for the medullary sheaths in the central nervous system of vertebrates is Weigert's stain (1884). The sections of material fixed in Müller's or Erlicki's solution and not yet turned green are placed at 35 to 40° for 1 to 2 hours (or according to Edinger for 24 hours at ordinary temperatures) in a solution of hematoxylin (¾–1 g. in 10 cc. alcohol and 90 cc. water) which is a few days old and therefore contains hematein; they become very black and must then remain in a mixture of borax (4 g.) potassium ferricyanide (5 g.) and water (200 cc.) until the gray matter has become yellowish. Flesch (1884) has modified this method by placing the sections first for a few minutes in a 0.5% chromic acid and then into hematoxylin. Another modification of this method originates with Pal (1886, 1887). He differs from Weigert only in treating the overstained sections with potassium hypermanganate (¼% solution) and then with oxalic acid and potassium sulfite (1 pt. to 200 of water); he thus obtains the gray matter entirely colorless while the white remains blue and the sections can then if desired be counterstained, for instance, with a carmin solution.

Hematoxylin With Copper

Böhmer (1865) was the earliest to try a copper salt (sulfate) as a mordant; while Cook (1879) gave the following formula:

Logwood extract	6 pts.
Alum	6 pts.
Copper sulfate	1 pt.
Water	40 pts.

All ingredients must be free from iron. Grind the alum, logwood extract and copper sulfate in a mortar; add sufficient water to form a paste; filter after 2 or 3 days. Add a crystal of thymol as a preservative. Dilute considerably for use.

Others, including Benda and Weigert, have developed procedures calling for mordanting in a solution of some copper salt. Today, however, such methods have largely been superceded by the iron-hematoxylin procedures.

Iron-Hematoxylin

In the iron-hematoxylin staining procedures an oxidation product is formed in the tissue, which according to Mayer, is a higher compound than hematein altho its composition is unknown. In these procedures the sections are ordinarily treated first with an iron salt, then stained with hematoxylin, and differentiated with an acid solution.

Benda (1886) stained with 1% aqueous hematoxylin after mordanting with concentrated ferric ammonium sulfate. Later (1893) he replaced this iron salt with Liq. ferri sulf. oxyd. of the German pharmacopoeia (diluted with two parts of water), which he applied to the sections 24 hours; then he stained with hematoxylin, washed and differentiated either in 30% acetic acid (or weaker) or in Liq. ferri sulf. oxyd. diluted 20 times.

M. Heidenhain (1892) placed sections for ½ to 3 hours in a 1½ to 4% solution of iron alum; then washed them in water and let them lie for half an hour in a 0.5% aqueous solution of hematoxylin, washed, and differentiated in the iron alum solution.

A great variety of similar formulae have since been proposed differing in the iron salt employed and in the method of application. Well known among them is that of Weigert's (see Mallory and Wright 8th Ed., p. 95) calling for iron sesquichloride as a mordant.

Hematoxylin With Other Metals

Molybdenum enters into a formula called for by Mallory (1891) who stained sections with an old mixture of hematoxylin with 1 part 10% phosphomolybdic acid, 6–10 parts chloralhydrate and 100 parts water.

Vanadium was employed by Wolters (1891) on celloidin sections of material fixed in Kultschitzky's fluid (potassium bichromate and copper sulfate in 50% alcohol) mordanted in the following:

10% vanadium chloride solution	1 part
8% aluminium acetate solution	4 parts

The sections were then stained in Kultschitzky's hematoxylin (1 g. in 100 cc. 2% acetic acid), and differentiated in acid alcohol (1 part HCl to 200 parts 80% alcohol). This proves a very useful nervous tissue stain.

Magnesia, platinic chloride, zinc sulfate and salts of various other metals have been employed as mordants for this dye. Noteworthy among such formulae is Mallory's phosphotungstic acid hematoxylin (1897):

Ammoniacal hematein	0.1 g.
Water	100 cc.
Phosphotunstic acid (crystals)	2 g.

Dissolve the hematein in a little water by the aid of heat and add it after cooling to the rest of the solution. Hematoxylin may be used instead of ammoniacal hematein, but requires 10 cc. of a 0.25% solution of potassium permanganate to ripen it.

This stain is extremely useful for various histologic and cytologic material. It is one of the most striking hematoxylin formulae yet developed, in the wealth of detail and contrasting color obtainable with it. Anyone glancing at a preparation thus stained and comparing it with one made by one of Böhmer's procedures will quickly decide that altho hematoxylin has long been a much prized stain, the early microscopists did not begin to realize its possibilities.

Recent Developments

The latest addition to the knowledge of hematoxylin and its behavior is in part the result of the shortage of this stain in America caused by the war. The first American hematoxylins were almost unrefined aqueous logwood extract. To improve the situation in his own laboratory, Dr. C. E. McClung manufactured a purified hematoxylin on a small scale which was quite satisfactory. The situation as it existed shortly after the war is discussed by him in a recent paper (1923). The manufacturing angle was discussed about this same time by Houseman and Swift (1920, 1925).

The realization that the dark colored hematoxylin then on the market was impure and hence unsatisfactory led to a demand on the part of biologists for a "white" hematoxylin. The American manufacturers tried to supply this literally, and as a result there was put on the market for a few years a product that had been bleached with sulfur dioxide. This bleached dye proved unsatisfactory as shown by McClung in the paper above cited. As a result the American hematoxylin now manufactured is no longer bleached, and is not quite so light colored as that which was sold about 1923; but it is rather purer and quite satisfactory as a stain.

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THE CHEMICAL ANALYSIS OF THIAZIN EOSINATES

W. C. HOLMES

(*Contribution No. 154 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washngton, D. C.*)

ABSTRACT—**The constituents of blood stains are normal eosinates of the thiazin series of dyes. Methods are outlined for the chemical examinations of blood stains which are based upon the complete reduction of all dye components present in buffered solutions of limited acidity, and the reduction of their thiazin components alone in strongly acid solutions. Chemical analysis should be supplemented by spectrophotometric analysis to obtain the most adequate characterization of the thiazin components of the eosinates.**

Both chemical and physical (spectrophotometric) methods are needed for the examination of Wright's stains and other blood stains of similar general type. Chemical methods were developed in the investigation reported in this paper. They will be utilized subsequently in obtaining reliable physical data.

Blood stains are usually prepared by the alkaline oxidation of aqueous solutions of methylene blue and the precipitation of the resulting mixture of the dye and its lower homologs by the addition of an aqueous solution of eosin. It would be anticipated that normal thiazin eosinates would be obtained, one molecule of eosin reacting with two thiazin molecules with the elimination of two molecules of sodium chloride as a soluble by-product. It is conceivable, however, that the eosinates might adsorb excess dyes present or, even, under favorable conditions, that a thiazin "bieosinate" might be formed thru the reaction of a molecule of eosin with a single thiazin molecule. It was considered essential to determine whether or not the nature of the precipitated stain was influenced by excess of either dye component, particularly since such stains are usually prepared in accordance with formulae which make no allowance whatever for the wide variations in actual dye content which may be encountered in the dyes employed.

To obtain information on this point, a duplicate series of precipitations was carried out with known quantities of dyes, in which eosin was added, respectively, to one (A), two (B), and four (C) molecular equivalents of methylene blue. The precipitated stains were filtered thru weighed Gooch crucibles, dried at 110°C., and weighed. Aliquots of the filtrates were examined for methylene blue and for total dyes by titration with titanous chloride under the

general conditions outlined below. The actual consumption of titanous chloride by methylene blue and by total dyes in the filtrates was checked against the theoretical consumption, calculated upon the known excess of dye present over the quantity required for the formation of the normal eosinate, and upon the quantities of eosinate present in solution as indicated by the observed deficiency in yield of precipitate. Table 1 shows this comparison. The calculations assume that the normal eosinate is the sole reaction product and that no appreciable quantities of soluble dyes are adsorbed under any circumstances. The checks obtained indicate that these assumptions are valid.

Variation in the proportions of the reacting dyes is without influence upon the character of the precipitated stain. The normal eosinate is obtained under all conditions. The yield of eosinate, however, varies considerably with conditions, and the maximum yield is obtained only when the correct proportions of dyes are employed for its formation. The eosinate is more soluble in a solution of methylene blue than in water and more soluble in a solution of eosin than in either.

The following methods are suggested for the chemical analysis of thiazin eosinates and blood stains in general.

Moisture is determined by drying to constant weight at 110°C.

The thiazin content is determined by dissolving a 0.2 g. -sample in 50 cc. of glacial acetic acid, adding 100 cc. of alcohol, heating to boiling and titrating with 1.0 /N titanous chloride solution in a current of carbon dioxide. Under these circumstances the thiazin present may be reduced and decolorized completely, whereas the eosin is entirely unaffected. The final disappearance of a blue green tint in the orange solution affords a satisfactory end point.

The total dye content is determined by dissolving a 0.2 g.-sample in 100 cc. of alcohol, adding 100 cc. of water and 50 cc. of a 20% Rochelle salt solution, heating to boiling and titrating under carbon dioxide. Under these conditions both the thiazin dye and the eosin are completely reduced and decolorized. The titanous chloride should be added slowly as the end point is neared. The final disappearance of the pink tint is taken as the end point.

In titrating both the thiazin and the total dye the analyst may prefer to run in a small excess of titanous chloride solution and determine the excess by back titration with a standard iron solution. In each instance the reduced thiazin present serves as an indicator, and the appearance of a blue green tint affords a very satisfactory end point.

TABLE 1. REACTION BETWEEN EOSIN AND METHYLENE BLUE.
Analytical data compared with the theoretical.

Sample*	Methylene blue	Eosin	Total volume	Theoretical eosinate	Precipitate	Eosinate in filtrate	Thiazin consumption of $TiCl_3$ in filtrate (cc. 10/N sol.) found	Thiazin consumption of $TiCl_3$ in filtrate (cc. 10/N sol.) calculated	Total dye consumption of $TiCl_3$ in filtrate (cc. 10/N sol.) found	Total Dye consumption of $TiCl_3$ in filtrate (cc. 10/N sol.) calculated
	Grams	*Grams*	*cc.*	*Grams*	*Grams*	*Grams*	*cc.*	*cc.*	*cc.*	*cc.*
A	0.04622	0.1000	200	0.0878	0.0431	0.0447	1.52	1.47	3.68	3.65
	"	"	"	"	0.0434	0.0444	1.48	1.46	3.64	3.64
B	0.09243	0.1000	"	0.1755	0.1508	0.0247	0.84	0.81	1.24	1.22
	"	"	"	"	0.1514	0.0241	0.80	0.79	1.20	1.19
C	0.09243	0.0500	"	0.0878	0.0601	0.0277	3.84	3.80	4.28	4.26
	"	"	"	0.0878	0.0600	0.0278	3.84	3.80	4.32	4.26

*In these three samples eosin was added respectively to one (A), two (B), and four (C) molecular equivalents of methylene blue.

The eosin content of the sample may be determined by means of a bromine determination but is much more conveniently calculated on the basis of the difference between the titanous chloride consumptions of total dye and of thiazin dye.

The methods outlined have been tested thoroly with a variety of thiazin eosinates prepared in this laboratory and have given very satisfactory results.

In applying them to the examination of blood stains of unknown origin and preparation the following considerations will enter. The ratio of the thiazin consumption of titanous chloride to the total dye consumption is precisely 2 to 3 for any thiazin eosinate, irrespective of the identity of the thiazin dye. Variation from this ratio in the titration values obtained with any stain sample is indicative of free thiazin dye or of free eosin, and the data will determine the identity and quantity of the dye in question. Total dye content can not be calculated from the titration values owing to the uncertainty respecting the exact character of the thiazin content. It may be obtained "by difference" by determining moisture (and salt, if present).

The character of the thiazin components of the eosinates in blood stains can be determined most adequately by means of spectrophotometric examination, but chemical analysis may be of some service. The total eosinate content of stains can be determined, as indicated, with fair precision, and the consumption of titanous chloride by eosinate can be measured accurately. With any stain it should be possible, accordingly, to determine the titanous chloride consumption per gram of eosinate, and this determination should throw light on the thiazin components. With methylene blue eosinate this consumption is 49.42 cc. 10/N titanium trichloride solution; with trimethyl thionin eosinate it is 50.58 cc.; with methylene violet eosinate it is 51.72 cc.; and with dimethyl thionin eosinate it is 51.81 cc.

A METHOD FOR MAKING ACETO-CARMIN SMEARS PERMANENT

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ABSTRACT.—**Anthers are collected and placed in a solution of 1 part acetic acid to 3 parts of absolute alcohol. The contents of the anther are squeezed out on a slide in a drop of Belling's iron-aceto-carmin solution and a cover glass placed over the drop. Care should be taken to remove all anther walls and flower parts. Heat the slide over an alcohol flame for a second, repeating 4 or 5 times. Place the slide in a petri dish filled with a 10% solution of acetic acid. When the cover glass has risen away from the slide gently remove the cover glass and place in a Coplin jar containing equal parts of alcohol and acetic acid. Likewise, place the slide in this solution. Run both cover and slide thru the following solutions: 1 part acetic acid to 3 parts absolute alcohol, 1 part acetic acid to 9 parts absolute alcohol, absolute alcohol and finally equal parts of absolute alcohol and xylol. Recombine the cover and slide in xylol-balsam directly from this solution.**

With the increasing use of Belling's iron-aceto-carmin method for making chromosome counts and its partial displacement of the tedious but permanent paraffin method has come the need for a record of such counts in the form of permanent preparations. One advantage of the aceto-carmin method is its speed; one disadvantage, a lack of permanency in the preparations. Altho several methods of making such preparations permanent have been suggested[1] the author has had more consistent results with the method to be outlined below.

Material is collected and placed immediately in a bottle containing 1 part of glacial acetic acid to 3 parts of absolute alcohol. Altho maize sporocytes have been kept in this killer for from several days to several weeks, the very best preparations were obtained from material that had been in killer from 12–24 hours. Over a short period of time, at least, this killer decidedly improves the appearance and stainability of the chromosomes in aceto-carmin preparations.

[1]Belling, J. The iron-aceto-carmin method of fixing and staining chromosomes. Biol. Bull., **50**, 160–162. 1926.

Heitz, E. Der Nachweis der Chromosomen. Vergleichende Studien über ihre Zahl, Grösse und Form im Pflanzenreich I. Zeit. f. Bot. **18**, 625–681. 1926.

Longley, A. E. Supernumerary chromosomes in Zea mays. J. Agr. Res.. **35**, 769–784. 1927.

For longer storage the material can be transferred directly from the killer to 70% alcohol, and aceto-carmin preparations can then be made at any later time (Fig. 1). Anthers can be transferred from the killer or 70% alcohol to a small drop of iron-aceto-carmin[2] solution. The contents of the anther should be carefully squeezed or teased out into this drop. At this point it is important to mention that all anther walls or flower parts, other than the sporocytes themselves, should be removed so that the cover-glass, when placed over the drop, will come into direct contact with the sporocytes. After the cover-glass has been gently placed over the material, the slide is

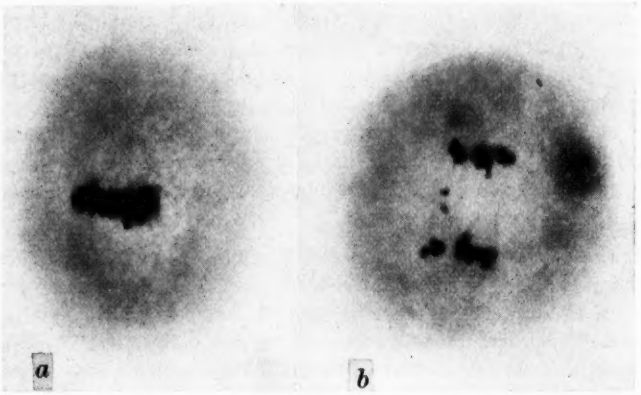

FIG. 1. Photomicrographs of microsporocytes of *Zea mays* from permanently mounted aceto-carmine smears: *a*, metaphase of first meiotic mitosis; *b*, anaphase of first meiotic mitosis.

held over an alcohol flame for about one second. This should be repeated 4 or 5 times until the cells are somewhat spread and flattened. Heating greatly increases the contrast between chromosomes and cytoplasm and brings about an adhesion of the sporocytes to the slide or cover-glass. Likewise, material that has been in killer or in alcohol for some time is very much improved by heating. The solution must not be allowed to boil! Experience will be required to determine the amount of heat necessary to give the best results.

Where a great many slides are to be studied during a season, and where it is neither necessary nor desirable to render permanent all

[2]The writer has employed certified carmine (NCa 2) in this technic.

the slides made, it is advisable to examine each slide soon after making in order that it may be placed in one of the following categories: (1) to be discarded after examination; (2) to be made permanent immediately; (3) to be ringed, so that the slide may darken or await further examination.

The next step depends upon whether the slide is to be made permanent immediately after examination or whether it has been ringed. If it is to be run up immediately, the immersion oil should be removed from the upper surface of the cover-glass. This should be done *very carefully*. The cover-glass must not be allowed to shift or the sporocytes will be pushed loose and will not stick to the glass. The slide is then placed in a petri dish filled with a 10% solution of acetic acid. The cover-glass will gradually rise somewhat from the slide, carrying some of the sporocytes with it. The cover-glass should be gently pushed over the edge of the slide so that it can be grasped with forceps. The cover-glass must not be pushed before enough solution has run between the slide and cover-glass to allow the latter to float freely. The sporocytes will be found to have stuck to both the slide and the cover-glass; consequently both must be handled in the solutions that follow. Carefully transfer the slide to a Coplin jar containing equal parts of acetic acid and absolute alcohol; likewise, transfer the cover-glass into the same solution. Then pass both thru the following solutions, a few minutes in each solution being sufficient: 1 part acetic acid to 3 parts absolute alcohol, 1 part acetic acid to 9 parts absolute alcohol, absolute alcohol, and finally equal parts of absolute alcohol and xylol. The slide and cover-glass are recombined directly from this solution in xylol-balsam. A pure xylol solution must not be used on fresh slides as it will cause a distortion of the sporocytes. To obtain the best results, the solutions should be frequently renewed. The slide is removed from the alcohol-xylol solution and a drop of fairly thick xylol-balsam is placed over the sporocyte region. The cover-glass is then placed face down on the drop of balsam. This last operation should be done rapidly to prevent absorption of moisture and consequent clouding of the medium.

If a slide is not to be run up immediately it should be ringed with some brittle, non-sticky medium such as paraffin. The following mixture[3] is preferred since it forms a perfect seal and is quite easily removed. Heat equal parts by weight of paraffin and gum mastic and stir thoroly. Allow to cool. This mixture can be applied to the edges of the cover by means of a heated wire. Bend the end of a

[3]Used for some time in the laboratory of Prof. Otto Rosenberg at Stockholm.

piece of wire so that it can be placed parallel to the long edge of the cover-glass. Heat this end of the wire in a flame and place in the paraffin-mastic mixture. Enough of the mixture will melt and remain on the wire so that it can be carried to the edge of the cover-glass where it will immediately solidify. The slide will keep for some time thus sealed but will gradually become dark. Likewise, in this strong acetic acid solution the chromosomes will show signs of disorganization. If a weaker solution of acetic acid is used this disintegration apparently does not proceed so rapidly (Randolph). If at a later time it is desired to run the slide into balsam, the seal must be removed. This, of course, must be done very gently so that the cover-glass will not move. The paraffin-mastic mixture will be found to chip away readily. First remove the seal carefully from 3 sides. When this has been done a weak spring clip should be placed over the cover and slide to keep the cover from moving when the seal is being removed from the fourth side. When the seal has been sufficiently removed so that the cover will float free in a solution, proceed as has been directed.

If the slide has become too dark, the carmin stain can be removed by allowing a solution of acetic acid to run under the cover. The slide is then held over an alcohol flame and gently heated until the slide is sufficiently destained.

A NEW STAINING DISH FOR HANDLING COVER-GLASS PREPARATIONS

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The fixing, staining, dehydrating and clearing of preparations, which are made, for reasons of convenience or necessity, on cover-glasses rather than slides, are operations with which almost all technical workers sooner or later have to deal. Such preparations are in constant use by protozoologists and are occasionally employed by cytologists[1] and specialists in several other lines of biological study. Nevertheless, up to the present time all sorts of inconvenient, make-shift devices have been used for handling the cover-glasses, usually resulting in the use of unnecessary amounts of reagents and general dissatisfaction with the added difficulties in manipulation.

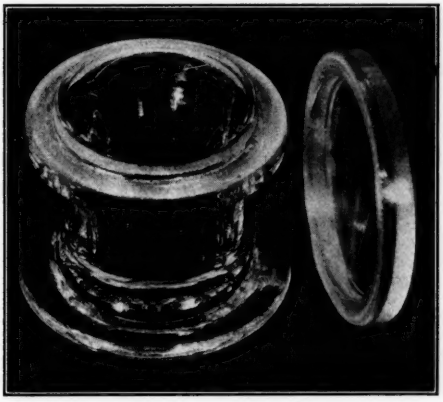

FIG. 1. Photograph of the staining dish; the lid is standing on edge at the right. (Four-fifths actual size.) Dimensions: height, 4 cm.; base 5 cm. in diameter.

In order to obviate these inconveniences I have recently designed a dish which permits the handling of large numbers of cover-glass preparations with a very small consumption of reagents and a minimum of trouble in the handling of individual cover-glasses. The dish is made of clear glass along the general lines of the Coplin and other

[1]In the method, for example, of mounting between two coverslips so that the preparation may be studied from both sides. See, for example: W. E. Agar, Quart. J. Micr. Sci., **57**, 1–44, 1911; and J. Gelei, Arch. f. Zellforschung, **16**, 88–170, 1921.

staining jars which are provided with grooves for handling microscopic slides. The new dish is shown in Fig. 1. It measures about 4 cm. high over all, and the base is 5 cm. in diameter. The interior contains grooves which permit the treatment of four cover-glasses at one time, or eight if they are placed back to back as is sometimes done in the case of slides. A cover with ground edge fits over the top of the jar and, being distinctly flanged, is not easily dislodged by accident. The base is unusually broad for the height of the jar, which is accordingly very stable. The grooves are calculated to accommodate cover-slips from ¾ to ⅞ of an inch square.

This dish is now in use in quantity in this laboratory and has proved exceptionally satisfactory. It should be found unusually convenient for the purpose for which it was designed. Thru the courtesy of the A. H. Thomas Co. of Philadelphia, Pa., this dish has now been placed in quantity production, and can be secured from that company under the name of the "Columbia cover-glass staining dish."

AN APPARATUS DESIGNED FOR HOLDING COVER SLIPS DURING FIXATION AND STAINING

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The need of an apparatus for fixing and staining cover slips upon which cell cultures are grown, led to the construction of the glass cover-slip holder here described. The holder is made with grooves on the sides and on the bottom into which the cover slips fit. The

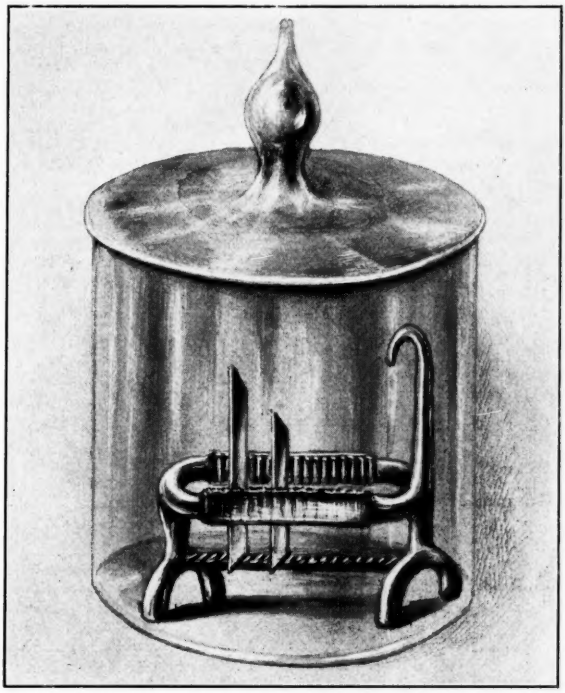

FIG. 1. Coverslip holder and staining jar for tissue culture and other coverslip staining.

holder illustrated is for eight cover-slips, but may of course be made for any number.

The tall handle makes it easy to transfer the holder from one staining or fixation jar to another. The size of the jar is conveniently 5.5 x 5.5 cm., and may therefore be used for the long, double size

cover slips by standing them upright in the holder. The jar is preferably one with a cover fitting tightly on a ground surface which may be made air-tight by vaseline. The solutions may so be kept at constant concentration.

While the holder may be used in any type of work, it has been designed especially for use with tissue cultures growing in plasma or hanging drops of culture media on cover slips.

The particular advantages of the holder are: 1) several preparations may be handled at one time thus making their treatment exactly alike, which is of great importance when control cultures are used; and 2) breakage, which often occurs when cover slips are changed singly, is avoided.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' coöperation is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order them direct from STAIN TECHNOLOGY, at a price of 6 cents each.

CHAZE, J. Sur le mode de formation et la détection des alcaloides dans la plantule de tabac. Bull. d'Histol. Appl., 5, 253. 1928.

Detection of nicotine and the mode of formation in the young plant was determined by the use of the two following methods. Plants, in the stage where the radicle is just leaving the teguments, were pressed lightly between a slide and cover slip and were treated with a dilute solution of neutral red. With this technic it was shown that the aleurone granules are in the process of transformation into semi-fluid vacuoles which are colored by neutral red. Since aqueous neutral red tends to dissolve the nicotine, use of Bouchardat's reagent is recommended for the demonstration of nicotine in plants at this stage of development. Combination of the two above methods clearly demonstrated that nicotine appears in the plant during the process of germination, in the interior of the vacuoles which arise from the hydration of the aleurone granules when these often still contain crystalloid protein.

NAN, YAO. Sur un fixateur cytologique particulièrement adapté aux tissues des insectes. Bull. d'Histol. Appl., 4, 71. 1927.

Fix in the following fixative 3 to 7 days:
NaCl 0.9%—86 cc.
Formol—14 cc.
Chromic acid 0.8%—20 cc.
Nitric acid—4 drops
Embed in paraffin, section and stain with iron-hematoxylin.

REICHARDT, H., and WETZEL, A. Paraffineinbettungsmethode nach vorhergegangener Zelloidindurchtränkung unter Vermeidung der härtenden Intermedien, Xylol, Benzol, Chloroform. Zeit. f. wiss. Mikr., 45, 476–479. 1928.

A modification of the Peterfis method for embedding hard or brittle objects is proposed using methyl benzoate in place of benzol for clearing the tissue. The tissue is treated in the usual way with absolute alcohol and then is placed in methyl benzoate until it becomes infiltrated and sinks. Treatment with 1% celloidin in methyl benzoate follows, tissue remaining in this for from two to five days according to its size. This is replaced by methyl benzoate to which paraffin chips have been added, the tissue remaining in this twelve to 24 hours at 40°C. Finally the objects are placed in melted paraffin at 50°C.; this is changed at least three times. Sections are cut with a knife that is not hollow ground. Avoiding the intermediate treatment with benzol prevents hardening of the object and also avoids shrinkage.

ERB, N. M. A rapid stain for direct miroscopic examination of milk. J. Lab. & Clin. Med., 14, 377. 1929.

Spread 0.01 cc. of milk over one square centimeter of a slide as in Breed method, dry in a warm place protected from dust and stain in the following solution:
Ether (sulphuric)—50 cc.
Methyl alcohol (absolute)—50 cc.
Methylene blue (certified)—0.59 g.
Stain for one minute, rinse in water, and dry.

GALESESCO, P., and BRATIANO, S. Coloration des graisses par l'extrait alcoolique de *Daucus carota*. Compt. Rend. Soc. Biol., **99**, 1460. 1928.

An alcoholic extract of carotin, the yellow pigment found in carrots, may be used in histology as a fat stain. Cut the cortical zone of carrots up into small pieces and immerse in 90 to 95% alcohol in a stoppered flask and put in a water bath for 2 hours. Allow to stand in a dark place for 8 to 10 days. Decant and filter the solution. At time of use dilute the solution with distilled water until the alcohol is from 60 to 80%. Frozen sections are cut from formol fixed tissue which has been washed in running water for 24 hours. Collect sections in water and then after placing for 5 minutes in 50 to 70% alcohol, stain in the alcoholic solution of carotin 6 to 24 hours. Put sections in 50 to 70% alcohol. Wash them and then stain the nuclei with Boetnner's hematoxylin. Wash in tap water and mount in glycerin jelly. Fat as well as lipoids (suprarenal), is stained golden yellow and shows up very well.

GOLDNER, J. Résultats obtenus chez la seiche par l'emploi de divers colorants vitaux Comp. Rend. Soc. Biol., **99**, 1323. 1928.

Three types of vital staining are discussed in connection with investigations on the cuttle fish. (1) Trypan blue and lithium carmin. These dyes stain fibrocytes, certain endothelial cells, adventicials and in addition a certain formation (the denticles of suckers on the tentacular arms) not stained by neutral red and rarely by acid fuchsin and light green. (2) Neutral red shows the vacuome contrasting with trypan blue. When the animals have stayed in sea water to which has been added a mixture of trypan blue and neutral red the contrast is sharper. Fibrocytes, endothelium of certain capillaries, adventicial cells, granulocytes, and horny denticles of the suckers are stained blue while the red stains only the vacuome and some small round elements of the blood system, some large amoebocytes and the branchial epithelium. (3) Indirect vital staining with acid fuchsin and light green by the method of Karczag and Paunz. In this method the dye is absorbed by different cells, changes to a colorless tautometric carbinol form, and is not regenerated until the sections are treated with dilute hydrochloric acid. This technic gives uncertain results with cuttle fish and cannot be used for vital staining of this material. (No information is given as to source or nature of dyes employed.)

HADJIOLOFF, A. Une modification rapide de la méthode de Weigert-Pal. Bull. d'Histol. Appl., **5**, 431–434. 1928.

Fix brain in skull by injection of formalin at the base of the neck. After 24 hours open the skull and continue the formalin fixation. Treat with potassium bichromate at 37 to 45° for several days according to the size: for one centimeter square and 3–5 m.m. thick, 3 or 4 days; for larger pieces, 4 to 8 days. Wash for 24 hours. Embed in paraffin. Section, and spread on slide by adding several drops of xylol; sections spread immediately. Remove xylol and add more, which is then replaced with alcohol, and finally with the dye solution. Stain warm for twenty to forty minutes, adding the stain with a pipette. Use Weigert's or Kultschistzky's hematoxylin. Wash in water and differentiate in 5% potassium permanganate for several seconds. Wash this off with a solution of 5% potassium bisulphite and 5% oxalic acid, made up, when used. Stain gray substance with Grübler-Holborn light green (1 to 5) for 4 to 10 minutes. Treat sections with alcohol, xylol and balsam.

HAUSDORF, G. Farbung zur Darstellung reifer Samenzellen im Hodenschnittpraparat. Zeit. f. wiss. Mikr., **44**, 327–328. 1927.

Since the usual methods give good results with the canals of the testicle but do not give a sharp picture of the ripe spermatozoa, the method was developed to supply this deficiency. Either alcohol or formalin fixation is satisfactory. Celloidin sections for staining must be fresh or the results will not be uniform. The thinnest possible, 10–20μ celloidin sections are placed directly on the slide and are covered with methyl alcohol for 20 minutes. Then blot with fiber-free filter paper. Drop on 0.5–1% victoria blue, cover with a Petri dish to prevent evaporation and stain twenty to twenty-four hours. (The writer gives no information as to the source or exact chemical nature of the dye employed.) After pouring off the dye, drop on officinal Lugol's solution and treat for two minutes. Sections will appear dark. Blot off this solution and differentiate the sections with anilin-xylol until they appear light blue and no more blue dye is washed out by fresh application of anilin-xylol. Treat sections with 80% alcohol; then with 60% alcoholic eosin for 30 seconds. Treat with the alcohols and

xylol and mount in balsam. Connective tissue is dull blue, blood corpuscles brown, spermatogonia dull red, heads of spermatozoa dark blue. For paraffin sections do not treat with methyl alcohol before staining but remove the paraffin in the usual way with xylol, treat with absolute alcohol and stain. Frozen sections cannot be used in this technic. These preparations may be photographed using a reddish light filter.

HOUCKE, E. Emploi des colorants mixtes en technique histologique. Comp. Rend. Soc. Biol., **99**, 783. 1928.

Five different combinations of acid and basic dyes are used in the same solution:

A) 1% toluidin blue, 10 parts; 1% orange G, 5 parts; distilled water, 85 parts. Allow this to stand 24 hours; collect the precipitate on a filter and allow to dry. Add 10 cc. of 99% methyl alcohol; this will saturate the alcohol with an excess of the stain.

B) Saturated aqueous thionin, 100 parts; 1% eosin, 17 parts. Make a 0.5% solution of the dried precipitate in 99% methyl alcohol.

C) 1% methylene blue, 10 cc.; 1% eosin, 11 cc.; distilled water, 79 cc. Make a 0.5% solution of the dried precipitate in 99% alcohol.

D) To 40 cc. of a 0.08% solution of azure-eosin add 48 drops (about 1.25 cc.) of 1% eosin. Allow filter paper to dry; cut it in strips and place for several days in a flask with 10 cc. of 99% methyl alcohol.

E) 1% aqueous toluidin blue, 10 parts; 1% eosin, 8 parts; distilled water, 82 parts. Make a 0.5% solution of the dried precipitate in 99% methyl alcohol.

Use 25 drops of A, 6–7 drops each of B, C, D, and E in 10 cc. distilled water with 2–3 drops of acetic acid (1–1000). Stain sections 24 hours, remove dye, wash quickly three times in absolute alcohol, then xylol and neutral dammar. Experimentation will determine the exact number of drops of 1–1000 acetic acid to add for the tissues being stained. (Writer does not specify dye-contents, or source from which stains were obtained; hence exact duplication of this very complicated formula may be difficult.)

HOUCKE, E. Emploi des mélanges de fuchsines et de blues basiques pour la coloration histologique. Comp. Rend. Soc. Biol., **99**, 786. 1928.

Staining by the following method may be used with Zenker-formol fixation, Bouin, and formol-alcohol, and is very good for red blood corpuscles and acidophil elements.

A) 1% aqueous acid fuchsin, 14 parts; 1% methylene blue, 22 parts. Pour the methylene blue solution into the acid fuchsin solution in a glass. Don't put a pipette or stirring rod into the mixture but allow it to stand 24 hours. Then pour off the liquid letting the glass drain. Dry the precipitate adhering to the glass and dissolve it in 20 cc. of 99% methyl alcohol.

B) Sat. aqueous thionin, 10 (cc.?); 1% acid fuchsin, 20 drops. Centrifuge, decant, drain and let the tube dry. Redissolve in 10 cc. of 99% methyl alcohol.

C) 1% aqueous toluidin blue, 11 cc.; 1% aqueous acid fuchsin, 5 cc. Treat as described for B.

To 10 cc. of distilled water add 10 drops each of A, B, and C, and 2–3 drops of acetic acid 1–100. Stain sections 2–3 hours; for formol fixed material ¼–½ hour may be sufficient. In general when the erythrocytes are bright red, the staining is sufficient. Wash three times in absolute alcohol, xylol, and mount in dammar. This general method may be used for any combination of an acid, and a basic dye. (Writer does not specify source or dye contents of the dyes; but gives directions for testing relative strengths in his solutions by "spotting out" on filter paper.)

HOUCKE, E. Emploi du mélange rhodamine-blue de méthylène dans la coloration des tissus splénique et lymphoïde. Comp. Rend. Soc. Biol., **99**, 788. 1928.

This method combines two basic dyes in one solution for double staining. By using one weakly basic dye and another of a strongly basic character, a dye bath may be obtained which stains the various tissue elements differentially and yet no precipitate is formed in aqueous solution. The method in this paper is usable for splenic or lymphoid tissue fixed in Dominici or Zenker-formol.

For tissues recently fixed the following procedure is used:

A) Sat.sol. methylene blue in 95% alcohol, 2 parts; sat. anilin water (diluted 1–2), 9 parts.

B) Solution A, 3 parts; 0.5% rhodamine B, 7 parts.

(No information is given as to source or dye content of these stains.)

Take 3 cc. of solution A. Stain the section 2–3 hours without addition of acid or alkali, wash quickly in absolute alcohol, treat with xylol and mount in neutral dammar. Basophile protoplasm is blue, chromatin blue violet, nucleoli red, connective tissue yellow red only slightly colored, muscle a deeper yellow red, erythrocytes bright red, acidophil granules of the leucocytes bright red, hyalin and granules of Russel bright red, nuclei of the small lymphocytes have a tendency to a violet appearance.

For tissue that has been kept in alcohol for a long time use undiluted anilin water in solution A and then proceed as in the above. The blue stains first and then the red, if the staining is too prolonged red staining will be too intense.

LILLIE, R. D. Eine Schnellmethode zur Toluidinblau-Schleimfarbung. Zeit. f. wiss. Mikr., **45**, 381. 1928.

The method is quick, gives permanent results, and either formol or sublimate material may be used. Frozen or paraffin sections are taken thru alcohol to water, stained for a minute in 0.2% aqueous toluidin blue (dye content or source of sample unspecified), washed quickly in water then with pure acetone, xylol and balsam. Old formol material causes the formation of a fine precipitate which can be removed by a short washing with 90% alcohol, followed by acetone and xylol. Mucus is stained red violet, cartilage blue violet, nuclei and bacteria deep blue; undecalcified bone is unstained, with blue bone cells, light violet Sharpy fibers, and bright blue border lamellae; decalcified bone is colored bright blue, red blood cells yellow or greenish yellow, and other tissue green, blue green, or blue.

OCHS, GEORG WILHELM. Über den Einfluss der Temperatur auf die Färbung von Blutausstrich-Präparaten. Folia Hematologica, **37**, 241–257. 1928.

An increase in methylene blue staining and a decrease of eosin staining occurs with increasing pH. A very good staining of neutraphilic granules is obtained with methylene blue at pH 6.8. With increase in temperature an increase in eosin staining was obtained and the difference in adsorption was noticed more in the changing of the color of the dye than by the change in intensity of the staining. With recognized exceptions there was also an increase in methylene blue staining with rising temperature. Results of experiments using Giemsa solution (Grübler) were like those obtained with methylene blue. The above results obtained with smears were confirmed by experiments using gelatin.

The staining solutions used were as follows: 1 part dye solution 1–1000 and 1 part buffer solution (Michaelis) pH 4.9, 6.0, 6.8. Slides were stained for one hour at 2°, 22° and 42° and were compared by examining with oil immersion using two microscopes with equal light sources.

VERNE, JEAN. Une nouvelle coloration élective de la myeline. Bull. d'Histol. Appl., **5**, 223–224. 1928.

This technic was inspired by that used by Feulgen in his work on plasmal and the reagent is the one used by Schiff (decolorized basic fuchsin) for the nucleal reaction. Nerve trunks not thicker than one centimeter are fixed for ten hours and not more than two days in one of the following fixatives.

1. Saturated aqueous sublimate, 30 parts; serum physiologique, 70 parts.
2. Platinic chloride, 1 g.; distilled water, 100 cc.

If desired the tissue may be preserved in a very dilute solution of the fixative used. Wash in running water for several minutes and then cut sections by the freezing method. Put the sections in 90% alcohol for ten minutes to remove bits of myelin detached by the razor. Wash in water and then put into the fuchsin reagent prepared as follows: To 200 cc. of a 1% basic fuchsin add 20 cc. of N/1 HCl and 1 g. of dry sodium bisulphite. (Information as to nature or source of dye is not given.) After twenty-four hours the solution is a yellow amber, ready for use. When the regions rich in myelin become intensely colored remove the section and rinse in 10 cc. HCl plus 10 cc. of liquid commercial sodium bisulphite plus 200 cc. of water. Renew the solution several times. Rinse in water and mount in sirup d'Apathy. Myelin is colored intense violet, gray matter and non-myelin region are uncolored.